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A P P L I C A T I O N

Of

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For

U N I T E D   S T A T E S   P A T E N T

On

OLIGONUCLEOTIDE THERAPEUTIC AGENT

501

AND

METHODS OF MAKING SAME

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BACKGROUND OF THE INVENTION

This invention relates generally to controlling

biologic functions, such as for antibiotic purposes, and more

particularly, to utilizing hybridization techniques of the type

having messenger ribonucleic acid attached to oligonucleotides

for _in vivo_ protein synthesis regulation.

In the field of pharmacology, the use of therapeutic

agents has long been recognized as an effective way to control

diseases. Such agents are often used in treating bacterial or

viral infections, chemical imbalances and the like, to cure,

or at least mitigate, the diseased state. Although researchers

occasionally discover new therapeutic agents after major break-

throughs have elucidated the molecular basis of a disease, mor

often they must rely on observing for antibiosis or modifying

the chemical structures of functionally related chemicals.

With respect to antibiotic agents, some are quite

effective at the outset, but over time many organisms become

resistant or totally immune to their action. Additionally, very

few effective anti-viral agents have ever been developed, and

without explicit, detailed knowledge of an infecting organism's

physiology, the development of new operative agents remains

haphazard.

Thus, there exists a definite need for a methodology

enabling the systematic design of new antibiotics and other

therapeutic agents that is versatile and inexpensive, yet

produces agents that are both extremely specific and effective.

The present invention fulfills these needs.

SUMMARY OF THE INVENTION

The present invention provides a methodology of
identifying and constructing therapeutic and other agents for
use in living organisms that substantially reduces the uncertain-
ty surrounding the development of new antagonists, significantly
increasing the scope of _materia medica_. Moreover, the agent
construction of the present invention lends itself readily to
simple manufacture, even in large quantities, is extremely
effective in use, and attains its improved results without undue
cross-reactions.

In a presently preferred embodiment of the invention,
by way of example and not necessarily by way of limitation, a
stabilized oligonucleotide, preferably in a phosphotriester
form, is provided having a base sequence substantially complemen-
tary to a portion of messenger ribonucleic acid coding for a
biological component of an organism. Due to the complement-
ary nature of the oligonucleotide and the messenger ribonucleic
acid, the two components can readily hybridize under appropriate
conditions to control synthesis of the organism's biological com-
ponent and, if the protein is vital to the organism's viability,
to act as an antibiotic.

A method, in accordance with the present invention,
of developing therapeutic agents may typically include the steps
of: providing a base sequence of an organism's nucleic acid that
contains at least a portion of the genetic information for a
biological component of the organism, and synthesizing an
oligonucleotide the sequence of which is derived from the base
sequence, for subsequent hybridization with the messenger

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ribonucleic acid specific for th biological component. The
biological component may be a vital protein, or simply a hormone
such as the gonadotropin, follicle stimulating hormone. The
order of the base sequence may be determined from deoxyribonu-
5   cleic acid (DNA) or ribonucleic acid (RNA), preferrably messenger
ribonucleic acid (mRNA). Alternatively, the desired oligonucleo-
tide base sequence may be determined from the biological component's
sequence, as when the biological component is a protein. The
preferred oligonucleotide has a minimum of about fourteen or
10   more bases, such as about twenty-three bases, and for increased
stability, may be transformed to a more stable form, such as a
phosphotriester form, to inhibit degradation during use.

To produce large quantities of the oligonucleotide,
it may be synthesized chemically, such as in automated machin s,
15   or inserted into a plasmid, such as pBR322, for cloning.
The plasmid insertion may be accomplished with linker base
sequences, such as GATTCGAATC or CTAAGCTTAG, which are susceptible
to degradation by Hind III restrictive nuclease or Alu I restric-
tion nuclease. When the order of the base sequence has not
20   been determined, the base sequence can be cloned and then
cross-hybridized against messenger ribonucleic acid from the
other sources to remove base sequences non-specific to the
target.

Another aspect of the present invention is a method
25   of selectively controlling activity of one or more biological
components in a cell without substantially interfering with th
activity of other biological components in the cell. The method
includes the steps of forming an oligonucleotide having a base

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sequnce substantially complementary to a portion of mRNA coding for the specific biological component, and introducing the oligonucleotide into the cell for hybridization with the selected mRNA. This causes blocking of tne translation of the mRNA into protein. The oligonucleotide may have at least about

5   fourteen bases or more, such as about twenty-three bases. The target mRNA can code for a protein, such as the hormone, follicle stimulating hormone. This hormone has an alpha and beta chain, and the oligonucleotide should be specific for the mRNA coding for the beta chain to avoid cross-reacting with other gonada-

10   tropin mRNA. A suitable oligonucleotide base sequence would b ACCACGCGR$_1$CCR$_2$ATGACGATGTG, wherein R$_1$ is G or T and R$_2$ is also G or T.

In accordance with another aspect of the present invention, a method is provided for inhibiting the infection of

15   a host organism by a foreign organism. This method entails isolating a base sequence containing at least a portion of the genetic information coding for a vital protein from the foreign organism's nucleic acid; synthesizing an oligonucleotide, the order of which is derived from the base sequence and substantially

20   complementary to the messenger ribonucleic acid coding for the protein; and treating the foreign organism with an effective amount of the oligonucleotide to hybridize with a portion of the messenger ribonucleic acid and block translation of the protein. The oligonucleotide, which can be a deoxyribonucleotide, can be

25   transformed to a more stable form, such as a phosphotriester form, to inhibit degradation, and the order of the sequence determined prior to its synthesis. Further, to increase the oligonucleotide's specificity, it may be cross-hybridized

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against mRNA from different organisms, such as the host organism, to remove non-unique oligonucl otide sequences.

It will be appreciated from the foregoing that the present invention satisfies a long existing need for improved methods of developing therapeutic agents for use in living organisms, and represents a significant advance over previously available methods, principally because it is very versatile, and yet provides a very specific agent against a biological component. Other aspects and advantages of the invention will become apparent from the following more detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram showing the central dogma of molecular biology;

FIG. 2 is a flow diagram showing normal translation of messenger ribonucleic acid (mRNA) into T protein, as well as a synthetic oligonucleotide of the present invention blocking translation of the T protein;

FIG.3 is a list of the viral deoxyribonucleic acid (DNA) code specific for SV-40 T protein, and the related mRNA and oligonucleotide;

FIG.4 is a flow diagram showing the construction of high yield plasmids containing the T protein oligonucleotid ;

FIG.5 is a flow diagram showing the use of restriction nucleases to cleave the plasmids to give a purified T protein oligonucleotide;

FIG. 6 is a flow diagram showing the treatment of a DNA sequence to form DNA polyphosphotriesters; and

FIG. 7 is a chart showing the partial amino acid sequence of follicle stimulating hormone, as well as the predicted mRNA sequence and related oligonucleotide family.

DETAILED DESCRIPTION OF THE INVENTION

Referring now to the drawings, and particularly to FIGS. 1 and 2, there is shown the so-called "central dogma" of the molecular biology of life. Basically, it is now accepted that deoxyribonucleic acid (DNA) carries the genetic code for almost all living organisms. The code exists in the form of an organized sequence of four nucleotide bases attached to a phosphorylated, deoxyribose sugar backbone. Generally, DNA exists in the form of a double strand helix formed of two oppositely directed strands, which are held together in an opposing manner by various weak forces.

A primary constituent of these weak forces are the so-called hydrogen bonds that exist between nucleotides on the opposing strands. The four bases, adenine (A), cytosine (C),

guanine (G), and thymine (T), form hydrogen bonds generally only
one fashion: A with T and C with G. Thus, by knowing th
sequence of one strand, the sequence of the second strand can
be readily determined.

5   Another aspect of the central dogma is that proteins
are produced indirectly from the DNA strand, through messenger
ribonucleic acid (mRNA). Apparently, mRNA, which has the same
structure as single stranded DNA except with a ribose backbone
and with uracil (U) replacing thymine, is transcribed directly
10   from one DNA strand and has an essentially opposite base
sequence, i.e., if a DNA strand sequence is 5´...ACGT...3´
the transcribed mRNA sequence is 3´...UGCA...5´.

           An additional aspect of the central dogma relates to
the translation of mRNA into proteins. Briefly, excluding
15   initiation sites and the like, each three nucleotide base group-
ing (triplet code) codes for one amino acid of a protein.
Therefore, by knowing the mRNA sequence of a protein, its amino
acid sequence can generally be determined. However, the
reverse is not true, that is, knowing the amino acid sequence
20   does not guarantee precise knowledge of the mRNA sequence.
This stems from the fact that there exist 64 (4^3) possible
triplet codes, yet there exists only about twenty amino acids,
allowing some amino acids to have multiple triplet codes.

           The similarity in the structure of DNA and mRNA
25   strands creates interesting effects. Most notably, if compl -
mentary DNA and RNA strands exist contemporaneously in a
solution, under certain established conditions the strands can

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anneal, forming hybrids. One important factor in proper annealing is the melting temperature, which can b calculated according to Britten et al, Methods of Enzymology 29:363 (1974).

5   In accordance with the present invention, a synthetic oligonucleotide having a base sequence capable of substantially matching that of a chosen mRNA is provided for hydridization with that mRNA. Once such a hybrid exists, the translation of the mRNA into protein becomes significantly inhibited. If the

10 inhibited protein is vital to an organism's survival, the organism's viability, i.e., either growth or continued life, is jeopardized. Importantly, the oligonucleotide can be designed specifically for the mRNA coding for just one protein, and should not cross-react with mRNA for other proteins.

15   The method of developing the oligonucleotide entails basically two steps. As described more fully below, a possible first step is to determine the appropriate sequence of the mRNA specific for the protein to be inhibited, and a second step is to manufacture an oligonucleotide complementary to the mRNA.

20 Once made, the oligonucleotide can be treated into a phospho-< triester form for increased stability.

      A variety of techniques exists for determining nucl ic acid base sequences. In many instances the sequence of the mRNA or the gene have been determined and published in the

25 biochemical literature. In fact, researchers have determined the complete nucleotide sequence for the SV-40 virus (Reddy et al, Science 200:494 (1978)). As is well known, an alternative

method entails isolating and purifying mRNA in sufficient quantities to permit sequencing studies, but this can prove difficult due to the relative instability and, in some cases, extreme rarity of many mRNA sequences.

5　　　　Still another method for determining nucleic acid base sequences requires resolving the amino acid sequence from the target protein. After determining the amino acid sequence of the target protein in purified form, a sequential degradation utilizing commercially available protein sequencors (e.g. from

10　Beckman Instruments, Fullerton, California) can be used to provide the amino acid sequence. Once this has been obtained, knowledge of the triplet code can be applied to give prospective base sequences. An example of such a process for the hormone glucagon can be found in Tullis et al, Biochemical and Biophysical

15　Research Communications 93:941 (1980).

Once the sequence of the appropriate nucleic acid and the desired mRNA sequence have been determined, an oligonucleo- tide, such as a deoxyribonucleotide, complementary to the mRNA can be constructed. A number of synthetic techniques are known,

20　most typical is the diester approach described by Agarwal et al, Nature 227: 27 (1970),and oligonucleotide synthesizers may be purchased commercially from Vega Biochemicals, P.O. Box 11648, Tucson, Arizona and Biologicals, Inc., Toronto, Canada.

25　　　　If the desired oligonucleotide sequence is unknown, a suitable oligonucleotide can be prepared as follows. After isolating mRNA from a target organism, multiple copies are made,

preferably in the form of DNA, so-called copy DNA (cDNA). This cDNA is then cross-hybridized against mRNA isolated from other organisms, and any cDNA hybridizing is removed. The remaining cDNA is specific only to the target organism, and can serve as the therapeutic agent.

5   In order to obtain a high degree of specificity, an oligomer of about fourteen or more residues can be constructed. Although shorter sequences will work, longer sequences provide higher specificity. This can readily be seen mathematically. Whereas a ten unit polymer chosen from four bases can have

10   4^{10} (1,048,576) ramdom combinations, a 20-unit polymer has 4^{20} random combinations, which equals 1.09×10^{12} (1,090,000,000,000).

In spite of the added difficulty in making oligonucleotides of twenty units in comparison to ten bases, it is

15   warranted because the exponential increase in complexity reduces undesirable cross-reactivity. It has been estimated that a mammalian cell contains about 2×10^{8} nucleotides of RNA complexity or, in other words, approximately 200 million nucleotides of unique sequence mRNA, which is equivalent to about 30,000

20   mRNA sequences. The probability that one of those sequences contains a randomly chosen 20-unit polymer is approximately one in fifty-five hundred. In comparison, a ten-unit polymer has about a one hundred and ninety to one chance for random cross-reaction.

25   The present invention is illustrated by, but not limited to, the following examples.

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EXAMPLE I

SV-40 virus manufactures a vital protein commonly
known as the "T protein" or "T antigen protein". As noted
earlier, the complete genetic code for the SV-40 virus has been
5   determined, and it is known that residues 5091 to 5071 on the
viral genome code for a portion of the T protein mRNA. The
sequence of these residues, the viral T protein mRNA sequence,
and the designed T protein specific oligonucleotide are shown
in Fig. 3. In this case, the T protein specific oligonucleotide
10   is complementary to the viral T protein mRNA, and identical to
the portion of the viral DNA code.

Prior to testing the effectiveness of the T protein
specific oligonucleotide _in vivo_, the oligonucleotide can be
mixed with total mRNA from an organism to check for cross-
15   reactivity. If it hybridizes, then a different portion of the
viral genome coding for the T protein should be utilized. Oth rwise,
the oligonucleotide is ready for further testing.

Further testing requires growth of S-40 virus. For
purposes of these experiments, SV-40 virus is grown and
20   titered in African green monkey cells, such as the cell line
BSC-1, according to Hopps et al, Journal of Immunology 91:416
(1963). The identity of the virus can be confirmed by the following
methods:

       a)   checking for tumor production after innoculation
25   of the newborn hamsters with the virus;

       b)   neutralization of the virus by anti-SV-40 anti-
serum; and

c)   reaction of the inf cted cells with anti-SV-40 T antig n directed antibodies prepared by standard techniques.

The isolation of SV-40 mRNA can be accomplished as follows. Total RNA is first obtained by the guanidine hydrochloric acid extraction procedure using glassware previously baked and treated with diethylpyrocarbonate to remove traces of RNase as taught by Cox et al, Methods in Enzymology 12B:120 (1968). The A+ RNA is isolated on oligo-dTcellulose, which can be obtained from Collaborative Research, Waltham, Massachusetts, or P. L. Biochemicals, Inc., Milwaukee, Wisconsin, utilizing the techniqu described by Bantle et al, Analytical Biochemistry 72:413 (1976). The RNA fractions are assayed for purity and intactn ss by electropheresis according to the method described in Bailey et al, Analytical Biochemistry 70:75 (1976). Also, the RNA can be assayed for translatability in the wheat embryo in vitro system described in Marcus, et al, Methods in Enzymology: 30:749 (1974). The in vitro translation products are monitored on sodium lauryl sulphate 9% polyacrylamide gels as described in Laemmeli, Nature 227:680 (1970).

This purified A+ mRNA containing the viral mRNA sequences can hybridize to the synthetic oligonucleotide at 37°C in 0.5 M sodium phosphate buffer, pH 6.8, containing 0.2% sodium lauryl sulphate. Solutions containing about 1 mg A+ mRNA and synthetic oligonucleotide at a concentration of about 100 ug/ml are heated to 100°C for 1-2 minutes, then cooled to 37°C and allowed to anneal. The extent of the hybridization reaction as a function of time may be monitored on a gel filtration column.

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Actually, while any theoretically suitable temperatur may be used for th hybrid formation, temperatures ranging from $0^{\circ}C$ to about $80^{\circ}C$ provide for good hybridization, but preferr d temperatures range from about $10^{\circ}C$ to about $40^{\circ}C$. Generally,

5   the optimal annealing temperature for the formation of specific hybrids is believed to be about $20^{\circ}C$ to $25^{\circ}C$ below their melting temperature. Synthetic oligonucleotides operating at $37.5^{\circ}C$ should thus be designed on the basis of their base sequence and length, such that the melting temperature is

10 between about $57^{\circ}C$ ana $62^{\circ}C$ when tested under approximately physiological conditions.

For hybridization testing the ratio of the synthetic oligonucleotide to its mRNA complement is generally about 30:1. Lower ratios are acceptable, however, sequences below about 3:1

15 can cause lower hybrid formation. Control reactions utilizing yeast RNA or globin mRNA can be used, and should show no det ctable hybrids, indicating hybridization specificity only to SV-40 mRNA. Also, thermal denaturation profile studies and comparison of the kinetics of hybridization can confirm that the synthetic

20 oligonucleotide reacts only with SV-40 mRNA sequences.

Once it is shown that the oligonucleotide hybridizes to the isolated SV-40 mRNA, _in vitro_ translation tests can be attempted utilizing the wheat embryo system (described previously) to show that the hybrid is not translated. Basically, upon

25 introduction of SV-40 mRNA into the wheat embryo system, the system produces large T antigen protein. However, when an

equal amount or more of synthetic oligonucleotide is also added
to th system, T antigen protein synthesis can be substantially
inhibited, without interference with synthesis of other SV-40
proteins whose mRNA was also introduced.

5   Testing of the oligonucleotide _in vivo_ can be accom-
plished by adding the oligonucleotide to cultures of cells
infected with SV-40. Synthesis of T antigen protein should be
inhibited signficantly in about six hours, and SV-40 growth
should be strongly inhibited within about 24 hours. The growth
10   of control cultures should be largely unaffected.

The synthetic oligonucleotide of the present invention
may be mass produced according to common cloning techniques, such
as those developed in the art to clone the gene for proinsulin.
Alternatively, the oligonucleotide can be chemically synthesiz d
15   in commercially available equipment (described previously).
Briefly, the cloning method entails enzymatic insertion of th
oligonucleotide into a bacterial gene carried on a larger piec
of DNA, known as a plasmid. The plasmid can be incorporated into
a suitable host bacteria, and multiple copies made as the
20   bacteria multiply as in Boyer and Cohen, U. S. Patent No.
4,237,224.

More particularly, and with reference to FIGS. 4 and
5, the cloning plasmid designated as pBR322, available from
Bethesda Research Labs, Inc., Rockville, Maryland, can be us d
25   to mass produce the T protein specific oligonucleotide. Using
standard techniques, the oligonucleotide is converted to

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double stranded form and then a terminal $5'PO_4$ is added to each of the 5' termini with polynucleotide kinase to permit subsequent joining through T-4 ligase. The reaction conditions for forming the 5' termini can be found in Richardson, Progress in Nucleic Acids Research 2:815 (1972).

5   After purification of the double stranded oligonucleo-
tide by chromatography on hydroxylapatite columns, it is inserted into the plasmid. Because the oligonucleotide is blunt ended, the plasmid should not have uneven or "sticky" ends. To remove sticky ends from the plasmid, S1 nuclease or other single
10   strand specific nucleases can be utilized. A general description of methods for using restriction nucleases can be found in Nathans and Smith, Annual Review of Biochemistry 44: 273 (1975).

           For best results, a linker system between the oligo-
nucleotide and the plasmid can be utilized, specifically a linker
15   having both Hind III and Alu I enzymatic cleaving sites. As seen in FIG. 4, one such linker has a sequence: 5'... CTAAGCTTAG...3'. This sequence represents a double stranded, bisymmetric molecul containing a recognition sequence both for Alu I (AGCT) and for Hind III (AAGCTT). Utilizing DNA ligase under standard con-
20   ditions, this molecule can be ligated to the oligonucleotide to form linker-oligonucleotide-linker molecules. Similarly, the linked oligonucleotide can be introduced into linearized blunt-ended, Hind III cleaved pBR322 carrier molecules.

           After ligation, the plasmid has resumed its covalently
25   closed circular configuration with the linker-oligonucleotide

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incorporated, all of which is shown in FIG. 5 as pT-protein oligonucleotide. Th recircularized plasmid is then used to transform a suitable bacterial host such as E. coli. The methods for transformation and selection of transformants are

5 known in the art and described in detail in Cohen and Boyer, U.S. Patent No. 4,237,224.

Once the transformed bacteria containing the ligated plasmid p-oligonucleotide have been grown to high density and produced large amounts of the ligated plasmid, the oligonucleo-

10 tide is ready for purification. After the plasmid has been removed from the mature cells, the plasmid is treated with appropriate restriction endonucleases. As illustrated in FIG. 5, the plasmid is first cleaved with Hind III to give various by-products, including linker-T-protein-oligonucleotide-linker

15 and fragments of the original plasmid. These are readily separated utilizing gel electrophoresis or high pressure liquid chromotography. Further cleavage of the isolated linker-oligonucleotide-linker with the endonuclease Alu I yields pure double stranded oligonucleotide and partially degraded linker.

20 These can also be separated based on their size differences.

As shown in FIG. 6, the oligonucleotide can then be modified to a nuclease resistant phosphotriester form utilizing the reaction described in Miller et al, Biochemistry 16:1988 (1977). Basically, the oligonucleotide is first acylated using

25 50% acetic anhydride-pyridine during an overnight incubation period. The product is precipitated and isolated from ether. The phosphotriester can then be formed utilizing 30% ethanol in anydrous 2, 6 lutidene (30%), NN-dimethyl formamide (30%) and

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p-tolu ne sulfonyl chlorid (17%), and r acting for about 6 hours. The prot cting acetyl groups are th n hydrolyzed by the addition of 0.5 volumes of concentrated ammonium hydroxide, followed by incubation for about 1 hour at 55°C. The final oligonucleotide product in the ethyl phosphotriester form can then be isolated on paper chromotography or high pressure liquid chromotography.

It is believed that transforming the oligonucleotide to a phosphotriester form will improve the oligonucleotide's stability in vivo due to an enhanced resistance against various degradative enzymes. However, the oligonucleotide will eventually degrade because of spontaneous de-ethylation, which leaves the molecule unprotected. Indeed, by controlling the initial level of ethylation, the in vivo degradation rate can be controlled. A further advantage of a phosphotriester form is believed to be an increase in the oligonucleotide's ability to penetrate a cell membrane.

EXAMPLE 2

A synthetic oligonucleotide capable of inhibiting the synthesis of follicle stimulating hormone (FSH), a protein hormone produced by the pituitary that functions in the maturation of ova in females and sperm cells in males, can also be constructed. It is known that FSH is composed of two chains, alpha and beta, the amino acid sequence of which has been determined for several animal species. Interestingly, the alpha chain of FSH is common to other gonadotropic hormones, including thyroid stimulating hormone, luteinizing hormone, and chorionic gonadotropin, whil th beta chain varies. Therefore, to

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s lectively shut off the synthesis of FSH without substantially affecting the other gonadotropins, th oligonucleotide must be specific for the mRNA coding for the beta chain.

5   The sequence of the beta chain amino acids 32 through 40 is shown in FIG. 7. As discussed earlier, it is possible to predict the mRNA base sequence for these amino acids, although not with absolute certainty. The points of uncertainty are indicated by the letter "X" in the predicted mRNA sequence. Thus, the resultant oligonucleotide family consists of eight possible 10   26 base sequences; the potential alternate bases are shown in parentheses below the primary base sequence.

By beginning with the projected mRNA sequence for the 33rd through 40th amino acids, it can be seen that four different 23 base oligonucleotides exist that could correspond 15   to the FSH mRNA. The sequences could be as follows, reading from the 5′ end: GTGTAGCAGTAGCCGGCGCACCA, GTGTAGCAGTATCCGGCGCACCA, GTGTAGCAGTAGCCTGCGCACCA, and GTGTAGCAGTATCCTGCGCACCA.

One of these four sequences should be precisely corr ct and thus able to hybridize fully with the FSH mRNA. To determine 20   the best sequence, a hybridization test against FSH mRNA, with subsequent purification on hydroxylapatite or other suitable column, can be performed as previously described. Once the b st sequence has been determined, it is placed in a plasmid or chemically synthesized, as described above, for bulk synthesis. 25   This oligonucleotide should substantially inhibit the synthesis of FSH _in vivo_.

From the foregoing, it will be appreciated that the pres nt invention provides a systematic method of designing new therapeutic agents for use in living organisms and that this method is versatile and inexpensive. Further, the oligonucleotide produced in accordance with the present invention is extremely effective and specific, enabling selective control of protein synthesis in a living organism.

While several particular forms of the invention have been illustrated and described, it will be apparent that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, it is not intended that the invention be limited, except as by the appended claims.